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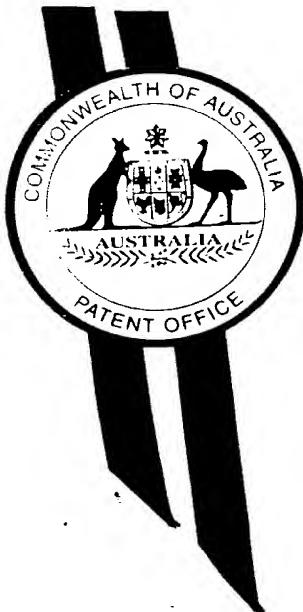
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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification as filed on 13 March 1997 in connection with Application No. PO 5662 for a patent by THE UNIVERSITY OF MELBOURNE and VICTORIAN DAIRY INDUSTRY AUTHORITY.

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AUSTRALIA
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PROVISIONAL SPECIFICATION

AUSTRALIAN	
PROVISIONAL No.	DATE OF FILING
P05662	13 MAR. 97
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Applicant(s):

THE UNIVERSITY OF MELBOURNE

VICTORIAN DAIRY INDUSTRY AUTHORITY

Invention Title:

CALCIUM PHOSPHOPEPTIDE COMPLEXES

The invention is described in the following statement:

CALCIUM PHOSPHOPEPTIDE COMPLEXES

The present invention relates to novel complexes in which amorphous calcium phosphates are stabilised by phosphopeptides. These complexes have anti-cariogenic effects, and may also be used as dietary supplements to increase calcium bioavailability and to heal or prevent diseases associated with calcium deficiencies. Methods of making the complexes of the invention and of treatment or prevention of dental caries, calcium malabsorption, and bone diseases are also provided.

Background

Dental Caries

Dental caries is initiated by the demineralisation of hard tissue on the teeth by organic acids produced from fermentation of dietary sugar by dental plaque odontopathogenic bacteria.

Even though the prevalence of dental caries has decreased through the use of fluoride in most developed countries, the disease remains a major public health problem. The estimated economic burden of treating dental caries in Australia in 1991 was \$471 million, being higher than that for other diet-related diseases including coronary heart disease, hypertension or stroke.

In developing countries where the availability of industrialised food products is increasing, prevalence of dental caries is also increasing. Recent studies have highlighted a number of socio-demographic variables associated with the risk of developing caries; high risk is associated with ethnicity and low socio-economic status. The level of high-risk individuals has remained constant even though the overall severity and prevalence of disease in the community has decreased. Dental caries is therefore, still a major public health problem, particularly in ethnic and lower socio-economic groups. This highlights the need for a non-toxic, anticariogenic agent that could supplement

the effects of fluoride to further lower the incidence of dental caries. An agent which would reduce the dose of fluoride required to reduce the incidence of caries would be particularly desirable in view of community anxiety about fluoride, and in view of the fact that fluorosis can develop even at currently used doses.

5 The food group most recognised as exhibiting anticaries activity is dairy products (milk, milk concentrates, powders and cheeses). US patent no. 5,130,123
10 discloses the component responsible for this anticariogenic activity as casein. However, the use of casein as an anticariogenic agent is precluded by adverse organoleptic properties and the very high levels required for activity.

15 In US patent no. 5,015,628, Reynolds disclosed that tryptic casein phosphopeptides were responsible for the anticariogenic activity, and that these peptides are Bos α_{s1} -casein X-5P (f59-79) [1], Bos β -casein X-4P (f1-25) [2], Bos α_{s2} -casein X-4P (f46-70) [3] and Bos α_{s2} -casein X-4P (f1-21) [4].

- 20 [1] Gln⁵⁹-Met-Glu-Ala-Glu-Ser(P)-Ile-Ser(P)-Ser(P)-
 Ser(P)-Glu-Glu-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-
 Gln-Lys⁷⁹. α_{s1} (59-79)
[2] Arg¹-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-
 Val-Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-
 Ser-Ile-Thr-Arg²⁵. β (1-25).
25 [3] Asn⁴⁶-Ala-Asn-Glu-Glu-Tyr-Ser-Ile-Gly-Ser(P)-
 Ser(P)-Ser(P)-Glu-Glu-Ser(P)-Ala-Glu-Val-Ala-Thr-
 Glu-Glu-Val-Lys⁷⁰. α_{s2} (46-70)
[4] Lys¹-Asn-Thr-Met-Glu-His-Val-Ser(P)-Ser(P)-
 Ser(P)-Glu-Glu-Ser-Ile-Ile-Ser(P)-Gln-Glu-Thr-
 Tyr-Lys²¹. α_{s2} (1-21)

30 In this patent, the amorphous forms of calcium phosphate are not disclosed. We have now found that Ca₃(PO₄)_{1.87}(HPO₄)_{0.2} x H₂O where $x \geq 1$ stabilised by the casein phosphopeptides is the most soluble, basic form of non-crystalline calcium phosphate and a superior form of

calcium phosphate which prevents caries and increase calcium bioavailability. Amorphous calcium phosphate (ACP) must be formed by careful titration of Ca ions (eg CaCl_2) and phosphate ions (eg Na HPO_4) while maintaining the pH above 7 (preferably 9.0) in the presence of the phosphopeptide. As the ACP is formed, the phosphopeptide binds to the nascent nuclei and stabilises the ACP as a phosphopeptide-ACP complex. Without the phosphopeptide, the ACP will precipitate out of solution and transform within minutes into the most stable calcium phosphate phase, crystalline hydroxyapatite (HA). HA, by being insoluble has limited anticariogenic activity and presents calcium in a poorly bioavailable form. The acidic phase of calcium phosphate CaHPO_4 , while certainly being more soluble than hydroxyapatite, is poorly bound by the phosphopeptide and poorly localised at the tooth surface and therefore also has limited anticariogenic activity. US patent 5,015,628 also does not disclose the unique amorphous calcium fluoride phosphate phase $\text{Ca}_8(\text{PO}_4)_F \times \text{H}_2\text{O}$ where $x \geq 1$ which we have now found to be stabilised by the phosphopeptides and localised at the tooth surface to provide superior anticaries efficacy.

Calcium Bioavailability

The availability of an adequate supply of calcium is essential for normal tooth and bone development and structure. A substantial body of evidence suggests that the dietary calcium intake during youth is an important determinant of peak bone mass and therefore of risk of osteoporosis in later life. Dairy products are the major source of dietary calcium in a typical Western diet, accounting for 60-75% of the usual dietary calcium intake. Not only is the calcium content of milk and milk products high, there is now considerable evidence that calcium from dairy products has increased bioavailability. The increased calcium bioavailability of dairy products has been attributed to the casein and casein phosphopeptides

released upon ingestion.

Summary of the Invention

In one aspect, the invention provides a stable calcium phosphate complex, comprising amorphous calcium phosphate or a derivative thereof stabilised by a phosphopeptide, wherein said phosphopeptide comprises the sequence - Ser(P)-Ser(P)-Ser(P)-Glu-Glu-.

The amorphous calcium phosphate (ACP) is preferably of the formula $[Ca_3(PO_4)_{1.87} (HPO_4)_{0.2} \times H_2O]$ wherein $x \geq 1$. The calcium phosphate derivative may be calcium fluoride phosphate having the formula $[Ca_8(PO_4)_5 F \times H_2O]$ wherein $x \geq 1$.

The phosphopeptide (PP) may be from any source; it may be obtained by tryptic digestion of casein or other phospho-acid rich proteins such as phosphitin, or by chemical or recombinant synthesis, provided that it comprises the core sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu-. The sequence flanking this core sequence may be any sequence. However, those flanking sequences in α_{s1} (59-79), β (1-25), α_{s2} (46-70) and α_{s2} (1-21) are preferred. The tryptic casein phosphopeptide denoted α_{s1} (59-79) is especially preferred. The flanking sequences may optionally be modified by deletion, addition or conservative substitution of one or more residues. The amino acid composition and sequence of the flanking region are not critical. The flanking regions stabilise the core sequence, but the core sequence alone is active. Moreover the flanking regions hinder access of the phosphate to the core sequence, although once binding takes place the complex is stabilised.

The complex formed preferably has the formula $[(PP)(CP)_n]_n$ where n is equal to or greater than 1, for example, 6. The complex formed may be a colloidal complex.

The phosphopeptide binds to the ACP cluster to produce a metastable solution in which growth of ACP to a

size that initiates nucleation and precipitation is prevented. In this way, calcium and other ions such as fluoride ions can be localised, for instance at a surface on a tooth to prevent demineralisation and prevent formation of dental caries.

5 Thus, in a second aspect, the invention provides a stable calcium phosphate complex as described above, which complex acts as a delivery vehicle that co-localises ions including, but not limited to calcium, fluoride and hydroxyl ions at a target site. In a preferred embodiment, 10 the complex is in a slow-release amorphous form that produces superior anti-caries efficacy.

In a particularly preferred embodiment of the 15 invention, the stable calcium complex is incorporated into dentifrices such as toothpaste, mouth washes or formulations for the mouth to aid in the prevention and/or treatment of dental caries or tooth decay. The calcium complex may comprise 0.05-50% by weight of the composition, preferably 1.0-50%. For oral compositions, it is preferred 20 that the amount of the CPP-ACP and CPP-ACFP administered is 0.05 - 50% by weight, preferably 1.0% - 50% by weight of the composition. The oral composition of this invention which contains the above-mentioned agents may be prepared 25 and used in various forms applicable to the mouth such as dentifrice including toothpastes, toothpowders and liquid dentifrices, mouthwashes, troches, chewing gums, dental pastes, gingival massage creams, gargle tablets, dairy products and other foodstuffs. The oral composition according to this invention may further include additional 30 well known ingredients depending on the type and form of a particular oral composition.

In certain highly preferred forms of the 35 invention the oral composition may be substantially liquid in character, such as a mouthwash or rinse. In such a preparation the vehicle is typically a water-alcohol mixture desirably including a humectant as described below.

Generally, the weight ratio of water to alcohol is in the range of from about 1:1 to about 20:1. The total amount of water-alcohol mixture in this type of preparation is typically in the range of from about 70 to about 99.9% by weight of the preparation. The alcohol is typically ethanol or isopropanol. Ethanol is preferred.

The pH of such liquid and other preparations of the invention is generally in the range of from about 5 to about 9 and typically from about 7.0 - 9.0. The pH can be controlled with acid (e.g. citric acid or benzoic acid) or base (e.g. sodium hydroxide) or buffered (as with sodium citrate, benzoate, carbonate, or bicarbonate, disodium hydrogen phosphate, sodium dihydrogen phosphate, etc).

In other desirable forms of this invention, the oral composition may be substantially solid or pasty in character, such as toothpowder, a dental tablet or a toothpaste (dental cream) or gel dentifrice. The vehicle of such solid or pasty oral preparations generally contains dentally acceptable polishing material. Examples of polishing materials are water-insoluble sodium metaphosphate, potassium metaphosphate, tricalcium phosphate, dihydrated calcium phosphate, anhydrous dicalcium phosphate, calcium pyrophosphate, magnesium orthophosphate, trimagnesium phosphate, calcium carbonate, hydrated alumina, calcined alumina, aluminum silicate, zirconium silicate, silica, bentonite, and mixtures thereof. Other suitable polishing material include the particulate thermosetting resins such as melamine-, phenolic, and urea-formaldehydes, and cross-linked polyepoxides and polyesters. Preferred polishing materials include crystalline silica having particle sized of up to about 5 microns, a mean particle size of up to about 1.1 microns, and a surface area of up to about 50,000 cm²/gm., silica gel or colloidal silica, and complex amorphous alkali metal aluminosilicate.

When visually clear gels are employed, a polishing agent of colloidal silica, such as those sold under the trademark SYLOID as Syloid 72 and Syloid 74 or under the trademark SANTOCEL as Santocel 100, alkali metal alumino-silicate complexes are particularly useful since they have refractive indices close to the refractive indices of gelling agent-liquid (including water and/or humectant) systems commonly used in dentifrices.

Many of the so-called "water insoluble" polishing materials are anionic in character and also include small amounts of soluble material. Thus, insoluble sodium metaphosphate may be formed in any suitable manner as illustrated by Thorpe's Dictionary of Applied Chemistry, Volume 9, 4th Edition, pp. 510-511. The forms of insoluble sodium metaphosphate known as Madrell's salt and Kurrol's salt are further examples of suitable materials. These metaphosphate salts exhibit only a minute solubility in water, and therefore are commonly referred to as insoluble metaphosphates (IMP). There is present therein a minor amount of soluble phosphate material as impurities, usually a few percent such as up to 4% by weight. The amount of soluble phosphate material, which is believed to include a soluble sodium trimetaphosphate in the case of insoluble metaphosphate, may be reduced or eliminated by washing with water if desired. The insoluble alkali metal metaphosphate is typically employed in powder form of a particle size such that no more than 1% of the material is larger than 37 microns.

The polishing material is generally present in the solid or pasty compositions in weight concentrations of about 10% to about 99%. Preferably, it is present in amounts from about 10% to about 75% in toothpaste, and from about 70% to about 99% in toothpowder. In toothpastes, when the polishing material is silicious in nature, it is generally present in amount of about 10-30% by weight. Other polishing materials are typically present in amount

of about 30-75% by weight.

In a toothpaste, the liquid vehicle may comprise water and humectant typically in an amount ranging from about 10% to about 80% by weight of the preparation.

5 Glycerine, propylene glycol, sorbitol and polypropylene glycol exemplify suitable humectants/carriers. Also advantageous are liquid mixtures of water, glycerine and sorbitol. In clear gels where the refractive index is an important consideration, about 2.5 - 30% w/w of water, 0 to

10 about 70% w/w of glycerine and about 20-80% w/w of sorbitol are preferably employed.

Toothpaste, creams and gels typically contain a natural or synthetic thickener or gelling agent in proportions of about 0.1 to about 10, preferably about 0.5 to about 5% w/w. A suitable thickener is synthetic hectorite, a synthetic colloidal magnesium alkali metal silicate complex clay available for example as Laponite (e.g. CP, SP 2002, D) marketed by Laporte Industries Limited. Laponite D is, approximately by weight 58.00%
20 SiO₂, 25.40% MgO, 3.05% Na₂O, 0.98% Li₂O, and some water and trace metals. Its true specific gravity is 2.53 and it has an apparent bulk density of 1.0 g/ml at 8% moisture.

Other suitable thickeners include Irish moss, iota carrageenan, gum tragacanth, starch,
25 polyvinylpyrrolidone, hydroxyethylpropylcellulose, hydroxybutyl methyl cellulose, hydroxypropyl methyl cellulose, hydroxyethyl cellulose (e.g. available as Natrosol), sodium carboxymethyl cellulose, and colloidal silica such as finely ground Syloid (e.g. 244).
30 Solubilizing agents may also be included such as humectant polyols such propylene glycol, dipropylene glycol and hexylene glycol, cellosolves such as methyl cellosolve and ethyl cellosolve, vegetable oils and waxes containing at least about 12 carbons in a straight chain such as olive
35 oil, castor oil and petrolatum and esters such as amyl acetate, ethyl acetate and benzyl benzoate.

It will be understood that, as is conventional, the oral preparations are to be sold or otherwise distributed in suitable labelled packages. Thus, a jar of mouthrinse will have a label describing it, in substance, as a mouthrinse or mouthwash and having directions for its use; and a toothpaste, cream or gel will usually be in a collapsible tube, typically aluminium, lined lead or plastic, or other squeeze, pump or pressurized dispenser for metering out the contents, having a label describing it, in substance, as a toothpaste, gel or dental cream.

Organic surface-active agents are used in the compositions of the present invention to achieve increased prophylactic action, assist in achieving thorough and complete dispersion of the active agent throughout the oral cavity, and render the instant compositions more cosmetically acceptable. The organic surface-active material is preferably anionic, nonionic or ampholytic in nature and preferably does not interact with the active agent. It is preferred to employ as the surface-active agent a detergitive material which imparts to the composition detergitive and foaming properties. Suitable examples of anionic surfactants are water-soluble salts of higher fatty acid monoglyceride monosulfates, such as the sodium salt of the monosulfated monoglyceride of hydrogenated coconut oil fatty acids, higher alkyl sulfates such as sodium lauryl sulfate, alkyl aryl sulfonates such as sodium dodecyl benzene sulfonate, higher alkylsulfo-acetates, higher fatty acid esters of 1,2-dihydroxy propane sulfonate, and the substantially saturated higher aliphatic acyl amides of lower aliphatic amino carboxylic acid compounds, such as those having 12 to 16 carbons in the fatty acid, alkyl or acyl radicals, and the like. Examples of the last mentioned amides are N-lauroyl sarcosine, and the sodium, potassium, and ethanolamine salts of N-lauroyl, N-myristoyl, or N-palmitoyl sarcosine which should be substantially free from soap or similar higher fatty acid material. The use of

these sarconite compounds in the oral compositions of the present invention is particularly advantageous since these materials exhibit a prolonged marked effect in the inhibition of acid formation in the oral cavity due to carbohydrates breakdown in addition to exerting some reduction in the solubility of tooth enamel in acid solutions. Examples of water-soluble nonionic surfactants suitable for use are condensation products of ethylene oxide with various reactive hydrogen-containing compounds reactive therewith having long hydrophobic chains (e.g. aliphatic chains of about 12 to 20 carbon atoms), which condensation products ("ethoxamers") contain hydrophilic polyoxyethylene moieties, such as condensation products of poly (ethylene oxide) with fatty acids, fatty alcohols, fatty amides, polyhydric alcohols (e.g. sorbitan monostearate) and polypropyleneoxide (e.g. Pluronic materials).

The surface active agent is typically present in amount of about 0.1-5% by weight. It is noteworthy, that the surface active agent may assist in the dissolving of the active agent of the invention and thereby diminish the amount of solubilizing humectant needed.

Various other materials may be incorporated in the oral preparations of this invention such as whitening agents, preservatives, silicones, chlorophyll compounds and/or ammoniated material such as urea, diammonium phosphate, and mixtures thereof. These adjuvants, where present, are incorporated in the preparations in amounts which do not substantially adversely affect the properties and characteristics desired.

Any suitable flavouring or sweetening material may also be employed. Examples of suitable flavouring constituents are flavouring oils, e.g. oil of spearmint, peppermint, wintergreen, sassafras, clove, sage, eucalyptus, marjoram, cinnamon, lemon, and orange, and methyl salicylate. Suitable sweetening agents include

sucrose, lactose, maltose, sorbitol, xylitol, sodium cyclamate, perillartine, AMP (aspartyl phenyl alanine, methyl ester), saccharine, and the like. Suitably, flavour and sweetening agents may each or together comprise from 5 about 0.1% to 5% more of the preparation.

In the preferred practice of this invention an oral composition according to this invention such as mouthwash or dentifrice containing the composition of the present invention is preferably applied regularly to the 10 gums and teeth, such as every day or every second or third day or preferably from 1 to 3 times daily, at a pH of about 4.5 to about 9, generally about 7.0 to about 9, for at least 2 weeks up to 8 weeks or more up to a lifetime.

The compositions of this invention can also be 15 incorporated in lozenges, or in chewing gum or other products, e.g. by stirring into a warm gum base or coating the outer surface of a gum base, illustrative of which may be mentioned jelutong, rubber latex, vinylite resins, etc., desirably with conventional plasticizers or softeners, 20 sugar or other sweeteners or such as glucose, sorbitol and the like.

In another embodiment, the complex of the invention is formulated to form a dietary supplement 25 preferably comprising 0.1-100% w/w, more preferably 1-50% w/w, most preferably 1-10% and particularly 2% w/w. The complex may also be incorporated into food products.

Accordingly, in a third aspect, the invention provides compositions including pharmaceutical compositions comprising the calcium complex as described together with a pharmaceutically-acceptable carrier. Such compositions may 30 be selected from the group consisting of dental, anti-cariogenic compositions, therapeutic compositions and dietary supplements. Dental compositions or therapeutic compositions may be in the form of a gel, liquid, solid, powder, cream or lozenge. Therapeutic compositions may 35 also be in the form of tablets or capsules.

In a fourth aspect, there is provided a method of treating or preventing dental caries or tooth decay comprising the step of administering a complex or composition of the invention to the teeth or gums of a 5 subject in need of such treatments. Topical administration of the complex is preferred.

In a fifth aspect, the invention relates to methods of treating one or more conditions related to calcium loss from the body, especially from the bones, 10 calcium deficiency, calcium malabsorption, or the like. Examples of such conditions include, but are not limited to, osteoporosis and osteomalacia. In general any condition which can be improved by calcium bioavailability is contemplated.

15 In a sixth aspect, the invention also provides a method of producing a stable complex of calcium phosphate as described above, comprising the step of

- (i) obtaining a solution of phosphopeptide having a pH of at least 9.0
- 20 (ii) admixing (i) with a solution comprising calcium and optionally fluoride at a pH of at least 9.0

- (iii) filtering the mixture resulting from step (ii) and
- 25 (iv) drying to obtain the said complex.

The complexes of the invention are useful as calcium supplements in subjects in need of stimulation of bone growth, for example subjects undergoing fracture repair, joint replacement, bone grafts, or cranofacial 30 surgery.

These complexes are also useful as dietary supplements in subjects who for any reason, such as dietary intolerance, allergy, or religious or cultural factors, are unable or unwilling to consume dairy products in an amount 35 sufficient to supply their dietary calcium requirements.

It will be clearly understood that, although this specification refers specifically to applications in humans, the invention is also useful for veterinary purposes. Thus in all aspects the invention is useful for 5 domestic animals such as cattle, sheep, horses and poultry; for companion animals such as cats and dogs; and for zoo animals.

Detailed Description of the Invention

The invention will now be described in detail by 10 way of reference only to the following non-limiting Examples.

Example 1 Preparation of CPP-ACP and CPP-ACFP

A. Preparation of CPP-ACP

A 10% w/v casein (Murray Goulburn, Victoria, 15 Australia) or caseinate solution was prepared at pH 8.0 and then digested with trypsin at 0.2% w/w of the casein for 2h at 50°C. After digestion the solution was adjusted to pH 4.6 by the addition of HCl and the precipitate removed by centrifugation or microfiltration. The supernatant or 20 microfiltrate was then adjusted to pH 9.0 with NaOH, then CaCl₂, and sodium phosphate pH 9.0 solutions were added slowly with constant agitation with the pH held constant at 9.0 by NaOH addition. CaCl₂, and sodium phosphate were added to the final concentrations of 100 mM and 60 mM 25 respectively. Following the addition of the calcium and phosphate solutions the solution was microfiltered through a 0.1 or 0.2 µm microfilter (ceramic or organic) and diafiltered with five volumes of distilled water. The retentate after diafiltration was spraydried to produce a 30 white powder that was 50% CPP and 40% ACP and residue water.

B. Preparation of CPP-ACFP

A 10% w/v casein or caseinate solution was prepared at pH 8.0 and then digested with trypsin at 0.2%

w/w of the casein for 2h at 50°C. After digestion the solution was adjusted to pH 4.6 by the addition of HCl and the precipitate removed by centrifugation or microfiltration. The supernatant or microfiltrate was then 5 adjusted to pH 9.0 with NaOH, then CaCl₂, and sodium phosphate pH 9.0 and NaF solutions were added slowly with constant agitation with the pH held constant at 9.0 by NaOH addition. CaCl₂, sodium phosphate and NaF were added to the final concentrations of 100 mM, 60 mM and 12 mM 10 respectively. Following the addition of the calcium, phosphate and fluoride solutions the solution was microfiltered through a 0.1 or 0.2 µm microfilter (ceramic or organic) and diafiltered with five volumes of distilled water. The retentate after diafiltration was spraydried to 15 produce a white powder that was 50% CPP and 40% ACFP and residue water.

The powdered CPP-ACFP was then reconstituted in distilled water to produce highly concentrated solutions. For example, a 10% w/v CPP -ACFP solution containing 640 mM 20 Ca, 400 mM phosphate and 80 mM F (1,520 ppm F⁻) at pH 9.0 has been prepared as well as a 20% CPP gel containing 1.28 M Ca, 800 mM phosphate and 160 mM F (3,040 ppm F⁻) at pH 9.0. This solution and gel exhibit a significantly greater anticariogenicity relative to the fluoride alone and 25 therefore are superior additives to toothpaste and mouthwash and for professional application to improve the efficacy of the current fluoride-containing dentifrices and professionally-applied products.

Example 2 Structural Studies of CCP-ACP

30 A. Structure and Interaction of CCP-ACP

Casein phosphopeptides containing the Ser(P) cluster, ie. the core sequence motif Ser(P)-Ser(P)-Ser(P)-Glu-Glu-, have a marked ability to stabilise calcium phosphate in solution. Solutions containing 0.1% w/v 35 α_{s1}(59-79) [1] at various pH, calcium and phosphate

concentrations, but constant ionic strengths were used to characterise the peptide's interaction with calcium phosphate. The peptide was found to maximally bind 24 Ca and 16 Pi per molecule as shown in Table 1.

The ion activity products for the various calcium phosphate phases [hydroxyapatite (HA); octacalcium phosphate (OCP); tricalcium phosphate (TCP); amorphous calcium phosphate (ACP); and dicalcium phosphate dihydrate (DCPD) were determined from the free calcium and phosphate concentrations at each pH using a computer program that calculates the ion activity coefficients through the use of the expanded Debye-Hückel equation and takes into account the ion pairs CaHPO_4^0 , $\text{CaH}_2\text{PO}_4^+$, CaPO_4^- and CaOH^+ the dissociation of H_2PO_4^- and H_2O and the ionic strength. The only ion activity product that significantly correlated with calcium phosphate bound to the peptide independently of pH was that corresponding to ACP $[\text{Ca}_3(\text{PO}_4)_{1.87}(\text{HPO}_4)_{0.12}\text{H}_2\text{O}]$ indicating that this is the phase stabilised by $\alpha_{s1}(59-79)$. The peptide $\alpha_{s1}(59-79)$ binds to forming ACP clusters producing a metastable solution preventing ACP growth to the critical size required for nucleation and precipitation. The binding of $\alpha_{s1}(59-79)$ to ACP results in the formation of colloidal complexes with the unit formula $[\alpha_{s1}(59-79)(\text{ACP})_n]$, where n is equal to or greater than one. It is likely that the predominant form is n = 6 as $\alpha_{s1}(59-79)$ cross-linked with glutaraldehyde in the presence of ACP runs as a hexamer on polyacrylamide gel electrophoresis. Interestingly, the synthetic octapeptide $\alpha_{s1}(63-70)$ AcGlu-Ser(P)-Ile-Ser(P)-Ser(P)-Ser(P)-Glu-GluNHMe only binds 12 Ca and 8 Pi per molecule i.e. $(\text{ACP})_4$ and the synthetic peptides corresponding to the N-terminus $\alpha_{s1}(59-63)$, Gln-Met-Glu-Ala-Glu and the C-terminus $\alpha_{s1}(71-78)$, Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln of $\alpha_{s1}(59-79)$ did not bind calcium phosphate as shown in Table 1. These results indicate that conformational specificity is essential for full ACP binding.

B. NMR Studies

Protein flexibility in solution is the outstanding characteristic to emerge from spectroscopy studies on proteins containing the Ser(P) cluster sequence (-Ser(P)-Ser(P)-Ser(P)-) such as phosvitin from egg yolk and phosphophoryn from tooth dentine. Phosphorylation appears to destabilise secondary and tertiary structure rather than promote higher levels of ordering. However, flexible phosphorylated sequences adapt more regular conformations when bound to calcium phosphate. Optical rotatory dispersion (ORD), circular dichroism (CD), hydrodynamic and ^{31}P nuclear magnetic resonance (NMR) measurements of the caseins all indicate that $\alpha_{\text{s}1}$ -casein and β -casein have a rather open structure in solution with many amino acid side chains exposed to solvent and relatively flexible. ^{31}P -NMR relaxation measurements indicate that Ser(P) residues are relatively mobile in β -casein.

We have demonstrated medium- and long-range nuclear Overhauser enhancements (noes) in 2D ^1H NMR spectra of $\alpha_{\text{s}1}(59-79)$ [1] in the presence of Ca^{2+} indicating a conformational preference. Two structured regions were identified. Residues Val72 to Val76 are implicated in a β -turn conformation. Residues Glu61 to Ser(P)67, which extend over part of the Ser(P) cluster motif -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- are involved in a loop-type structure. 2D NMR studies on β -casein(1-25) in the presence of calcium have shown a medium range noe in the -Ser(P)¹⁷-Ser(P)-Ser(P)-Glu²¹- motif region between the COH of Ser(P)¹⁸ and NH of Glu²⁰. Further medium range noes include one between the COH of Ser²² and NH of Thr²⁴. Evidence from the ^1H NMR spectra of $\alpha_{\text{s}2}$ -casein(1-21) [4] have shown that several residues including those around the -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- are perturbed. Furthermore, there are medium range noes between NH of Ser(P)⁸ and NH of Glu¹⁰. This is yet another example of a medium range noe in the -

Ser(P)-Ser(P)-Ser(P)-Glu-Glu- motif. Other examples of medium range nOes include that between the NH of Ile¹⁴ and NH of Ser(P)¹⁶.

In summary the NMR data indicates that
5 preferred conformations exist for these peptides in the presence of calcium ions. Molecular modeling of both $\alpha_{s1}(59-79)$ and $\beta(1-25)$ using the constraints derived from the NMR spectroscopy have indicated that the peptides adopt conformations that allow both glutamyl and phosphoseryl 10 side chains of the cluster motif -Ser(P)-Ser(P)-Ser(P)-Glu-Glu to interact collectively with calcium ions of the ACP.

The relationship between CPP structure and interaction with amorphous calcium phosphate was investigated using a series of synthetic peptide homologues and analogues indicated in Table 1. These studies showed 15 that the cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu was mainly responsible for the interaction with ACP and that all three contiguous Ser(P) residues are required for maximal interaction with ACP.

TABLE 1.
Calcium Phosphate Binding by CPP and Synthetic Homologues and Analogues

	V_{Ca} mol/mol	V_{Pd} mol/mol	Ca/P
$\Sigma\Sigma EE$	9	6	1.5
$S\S\S EEE$	2	1	2.0
$E\S\S EEE$	2	1	2.0
$D\S\S EEE$	2	1	2.0
$000EEE$	9	6	1.5
$S00EEE$	2	1	2.0
$A\S A E$	0	0	
$I A A E A$	0	0	
$E A I A A E A$	0	0	
$A A A Z A E$	0	0	
$A \Delta A S A A E$	2	1	1.5
$A \Delta A S A A A E$	6	4	1.5
$\alpha_{\text{S1}} (59-79)$	QMEAE Σ IV Σ EEIVPN Σ VEQR	24	1.5
$\alpha_{\text{S1}} (63-70)$	E Σ I Σ EE Σ EE	12	1.5
$\alpha_{\text{S1}} (66-70)$	Σ Σ EE	9	1.5
$\alpha_{\text{S1}} (71-78)$	IVPN Σ VEQR	0	0
$\alpha_{\text{S1}} (59-63)$	QMEAE	0	0
$\beta (1-25)$	RELEELNVPG E IVE E ESITR	24	1.5
$\beta (14-21)$	Σ Σ EE	12	1.5
		8	

$\Sigma = \text{Ser}(P)$, $\theta = \text{Thr}(P)$, $E = \text{Glu}$, $D = \text{Asp}$, $S = \text{Ser}$, $A = \text{Ala}$, $I = \text{Ile}$, $\text{Q} = \text{Gln}$, $M = \text{Met}$, $V = \text{Val}$,
 $P = \text{Pro}$, $K = \text{Lys}$, $L = \text{Leu}$, $T = \text{Thr}$, $G = \text{Gly}$ and $R = \text{Arg}$.

Example 3 — Structural Studies Using Hydroxyapatite (HA)

Similarly, we investigated the adsorption of the CPP and synthetic homologues and analogues onto HA (Table 2). These data also confirm that the Ser(P) cluster sequence is the major determinant for high affinity binding and that all three contiguous Ser(P) residues are essential as loss of any one, even when substituted with a Glu or Asp, resulted in a considerably lower affinity constant K as shown in Table 2.

Table 2. CPP and Synthetic Peptide binding to HA at 37°C

		K ml/μmol	N μmol/m ²	Molecul ar Area nm ²
$\alpha_{\text{a}1}$ (59-79)	QMEAE Σ I Σ E Σ IVPN Σ VEQK	41.5	0.35	4.75
$\alpha_{\text{a}1}$ (63-70)	E Σ LEE	10,370	0.47	3.56
$\alpha_{\text{a}1}$ (66-70)	Σ LEE	12,845	0.52	3.27
$\alpha_{\text{a}1}$ (71-78)	IVPN Σ VEQK	-	-	-
$\alpha_{\text{a}1}$ (59-63)	QMEAE	-	-	-
Σ LEE		12,845	0.52	3.27
Σ LEE		1,513	0.96	1.74
Σ LEE		6,579	0.81	2.04
θ θEE		12,234	0.51	3.27
TθEE		1,013	0.55	3.03
θTθEE		837	0.44	3.77
θTEE		1,799	0.46	3.61

$\Sigma = \text{Ser}(P), \theta = \text{Thr}(P)$

Interestingly, repeating these HA adsorption experiments with salivary coated HA (sHA) revealed that the Ser(P) cluster motif was still the major determinant for adsorption although the affinities of the peptides for the sHA was slightly reduced by the presence of the salivary proteins. These results suggest that the predominant interaction of the CPP with pellicle and plaque is likely to be electrostatic and mediated by the Ser(P) cluster motif of the CPP.

We have also studied the docking of the peptide Ser(P)-Ser(P)-Ser(P)-Glu-Glu- onto three crystallographic planes of HA, {100}, {010} and {001} using computer simulation techniques and the unit cell coordinates of synthetic HA. These simulation studies revealed that the peptide -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- is more likely to bind to the {100} surface, followed by the {010} surface. The Ser(P) cluster motif can therefore bind to both {100} and {010} surfaces thus allowing deposition of calcium, phosphate and hydroxyl ions on the {001} surface enabling growth of the HA crystal along the c-axis only. These results therefore can now explain the c-axis growth of HA crystals in enamel and dentine. Detailed examination of the computer simulation data shows that the -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- conformer with the greatest relative binding energy is positioned on the HA surface such that the carboxyl groups of the glutamyl residues and the phosphoryl groups of the phosphoseryl residues are in proximity to the HA surface with maximal contact between these groups and surface calcium atoms.

Example 4. Anticariogenic Activity of CPP-ACP in Human *in situ* Studies.

The ability of the 1.0% w/v CPP-ACP pH 7.0 solution to prevent enamel demineralisation was studied in a human *in situ* caries model. The model consists of a removable appliance containing a left and right pair of

enamel slabs placed to produce a plaque retention site. The inter-enamel plaque that developed (3-5 mg) was bacteriologically similar to normal supragingival plaque. On frequent exposure to sucrose solutions over a three week period, the increase in levels of mutans streptococci and lactobacilli and in sub-surface enamel demineralisation resulted in the formation of incipient "caries-like" lesions.

Two exposures of the CPP-ACP solution per day to the right pair of enamel slabs for 12 subjects produced a $51\% \pm 19\%$ reduction in enamel mineral loss relative to the left-side, control enamel. The plaque exposed to the CPP-ACP solution contained $78 \pm 22 \mu\text{mol/g}$ calcium, $52 \pm 25 \mu\text{mol/g P}_i$ and $2.4 \pm 0.7 \text{ mg/g CPP}$ compared with $32 \pm 12 \mu\text{mol/g}$ calcium and $20 \pm 11 \mu\text{mol/g P}_i$ in the control plaque. The level of the CPP was determined by competitive ELISA using an antibody that recognises both $\alpha_{s1}(59-79)$ and $\beta(1-25)$. Electron micrographs of immunocytochemically stained sections of the plaque revealed localisation of the peptide predominantly on the surface of microorganisms but also in the extracellular matrix.

Although these results indicate that CPP are incorporated into developing dental plaque, the actual level determined by ELISA would not be a true representation of that incorporated due to the breakdown of the CPP in plaque through the action of phosphatase and peptidase activities. The incorporation of the CPP-ACP in the plaque resulted in a 2.4 fold increase in the plaque calcium and a 2.6 fold increase in plaque P_i with a Ca/P_i ratio consistent with ACP.

Example 5. Anticariogenic Potential of the CPP-ACP in a Mouthwash Study.

A clinical trial of a mouthwash used thrice daily containing 3.0% CPP-ACP pH 9.0 showed that the calcium content of supragingival plaque (lower anterior teeth

excluded) increased from $169 \pm 103 \mu\text{mol/g}$ dry weight to $610 \pm 234 \mu\text{mol/g}$ after use of the mouthwash for a three day period, and inorganic phosphate increased from $242 \pm 60 \mu\text{mol/g}$ dry weight to $551 \pm 164 \mu\text{mol/g}$. These post-mouthwash levels of calcium and inorganic phosphate are the highest ever reported for non-mineralised supragingival plaque.

Without wishing to be bound by any proposed mechanism for the observed advantages, it is believed that the mechanism of anticariogenicity for the CPP-ACP is the incorporation of amorphous calcium phosphate in plaque, thereby depressing enamel demineralisation and enhancing remineralisation. In plaque, CPP-ACP would act as a reservoir of calcium and phosphate, buffering the free calcium and phosphate ion activities thereby helping to maintain a state of supersaturation with respect to tooth enamel. The binding of ACP to CPP is pH dependent with very little bound below pH 7.0.

Example 6. Remineralisation of Enamel Lesions by CPP-ACP.

An in vitro enamel remineralisation system was used to study remineralisation of artificial lesions in human third molars by CPP-ACP solutions. Using this system, a 1.0% CPP-ACP solution replaced $56 \pm 21\%$ of mineral lost. A 0.1% CPP-ACP solution replaced $34 \pm 18\%$ of mineral lost. A further number of solutions containing various amounts of CPP (0.1-1.0%), calcium (6-60 mM) and phosphate (3.6-36 mM) at different pH values (7.0-9.0) were prepared. The associations between the activities of the various calcium phosphate species in solution and the rate of enamel lesion remineralisation for this series of solutions were then determined.

The activity of the neutral ion species CaHPO_4° in the various remineralising solutions was found to be highly correlated with the rate of lesion remineralisation. The diffusion coefficient for the remineralisation process was

estimated at $3 \times 10^{-10} \text{ m}^2\text{s}^{-1}$ which is consistent with the coefficients of diffusion for neutral molecules through a charged matrix. The rate of enamel remineralisation obtained with the 1.0% CPP-ACP solution was $3.3 \times 10^{-2} \text{ mol HA/m}^2/10 \text{ days}$ which is the highest remineralisation rate ever obtained. Calcium phosphate ions, in particular the neutral ion pair CaHPO_4° , after diffusion into the enamel lesion, will dissociate and thereby increase the degree of saturation with respect to HA. The formation of HA in the lesion will lead to the generation of H_3PO_4 , which being neutral itself, will diffuse out of the lesion down a concentration gradient.

The results indicate that the CPP-bound ACP, CPP $[\text{Ca}_3(\text{PO}_4)_{1.87}(\text{HPO}_4)_{0.2} \cdot x\text{H}_2\text{O}]$, acts as a reservoir of the neutral ion species, CaHPO_4° that is formed in the presence of acid. The acid can be generated by dental plaque bacteria; under these conditions, the CPP-bound ACP would buffer plaque pH and produce calcium and phosphate ions, in particular CaHPO_4° . The increase in plaque CaHPO_4° would offset any fall in pH thereby preventing enamel demineralisation. Acid is also generated in plaque as H_3PO_4 by the formation of HA in the enamel lesion during remineralisation. This therefore explains why the CPP-ACP solutions are such efficient remineralising solutions as they would consume the H_3PO_4 produced during enamel lesion remineralisation generating more CaHPO_4° thus maintaining its concentration gradient into the lesion. These results are therefore consistent with the proposed anticariogenic mechanism of the CPP being the inhibition of enamel demineralisation and enhancement of remineralisation through the localisation of ACP at the tooth surface.

Example 7. CPP-ACFP Mouthwash Study.

A mouthwash study was conducted to determine the ability of a 3.0% CPP-ACFP mouthwash used thrice daily to increase supragingival plaque calcium, inorganic phosphate

and fluoride ions. The 3.0% CPP-ACFP solution used as a mouthwash for four days contained 192 mM bound calcium ions, 120 mM bound phosphate ions and 24mM (456 ppm) bound F ions stabilised by CPP. The use of the mouthwash resulted in a 5 1.9 fold increase in plaque calcium, a 1.5 fold increase in plaque phosphate and a dramatic 18 fold increase in plaque fluoride ion as shown in Table 3.

Table 3. Effect of CPP-ACFP on Plaque, Ca, Pi and F Levels

	Ca μmol/g	Pi μmol/g	F μmol/g
Control	177 ± 53	306 ± 82	1.1 ± 0.9
3% CPP - ACFP	336 ± 107	471 ± 113	19.9 ± 14.1
1000 ppm F	158 ± 54	287 ± 29	1.9 ± 1.0
3% CacPP	193 ± 56	343 ± 102	1.5 ± 0.8

Although these marked increases in plaque calcium, phosphate and fluoride were found, dental calculus was not observed in any of the subjects, suggesting that the plaque calcium fluoride phosphate remained stabilised as the amorphous phase by the CPP and did not transform into a crystalline phase. These increases in the supragingival plaque levels of Ca, phosphate and fluoride ions produced by CPP-ACP are markedly greater than those obtained in a similar study using CaCPP and 1000ppm F (MFP and NaF) toothpastes twice daily for a similar time period as indicated in Table 1. These results show a marked synergistic effect between fluoride ions and the CPP-ACP. This is particularly advantageous in view of the fact that the level of fluoride in oral compositions such as toothpaste can then be reduced, resulting in cost savings and lowered risk of fluorosis for individuals living in high-fluoride areas.

Example 8. Interaction of CPP-ACP with Fluoride

An synergistic anticariogenic effect of the 1.0% CPP-ACP together with 500 ppm F⁻ was observed in a rat caries model. Analysis of the solution containing 1.0% CPP, 60 mM CaCl₂, 36 mM sodium phosphate and 500 ppm F (26.3 mM NaF) pH 7.0 after ultrafiltration revealed that nearly half of the fluoride ion had incorporated into the ACP phase stabilised by the CPP to produce an amorphous calcium fluoride phosphate phase of composition Ca₈(PO₄)₅F.xH₂O, with 24 Ca, 15 PO₄ and 3F molecules per CPP molecule.

Without wishing to be limited by any proposed mechanism for the observed beneficial effect, we consider that the anticariogenic mechanism of the CPP-ACP is the localisation of ACP at the tooth surface such that in the presence of acid, the ACP dissociates to release Ca and phosphate ions increasing the degree of saturation with respect to HA preventing enamel demineralisation and

promoting remineralisation. The anticariogenic mechanism of fluoride is the localisation of the fluoride ion at the tooth surface, particularly in plaque in the presence of Ca and phosphate ions. This localisation increases the degree
5 of saturation with respect to fluorapatite (FA) thus promoting remineralisation of enamel with FA . It is clear that for the formation of FA $[Ca_{10}(PO_4)_6F_2]$, calcium and phosphate ions must be co-localised in plaque at the tooth surface with the fluoride ion. The synergistic
10 anticariogenic effect of CPP-ACP and F is therefore attributable to the localisation of ACFP at the tooth surface by the CPP which in effect would co-localise Ca, Pi and F.

This was demonstrated in the mouthwash study
15 described in Example 7.

Metastable solutions of the CPP at pH 7.0 have been prepared containing amorphous calcium fluoride phosphate at remarkably high concentrations. For example, a 10% w/v CPP -ACFP solution containing 640 mM Ca, 400 mM
20 phosphate and 80 mM F (1,520 ppm F⁻) at pH 7.0 has been prepared as well as a 20% CPP gel containing 1.28 M Ca, 800 mM phosphate and 160 mM F (3,040 ppm F⁻) at pH 7.0. This solution and gel exhibit a significantly greater anticariogenicity relative to the fluoride alone, and
25 therefore are superior additives to toothpastes and mouthwash and for professional application to improve the efficacy of the current fluoride-containing dentifrices and professionally-applied products.

Specific examples of formulations containing the
30 complexes of the invention are provided below.

Example 9 Toothpaste formulations containing CPP-ACFP

Formulation 1

<u>Ingredient</u>	<u>% w/w</u>
Dicalcium phosphate dihydrate	50.0
Glycerol	20.0

	Sodium carboxymethyl cellulose	1.0
	Sodium lauryl sulphate	1.5
	Sodium lauroyl sarcosinate	0.5 -
	Flavour	1.0
5	Sodium saccharin	0.1
	Chlorhexidine gluconate	0.01
	Dextranase	0.01
	CPP-ACFP	1.0
	Water	balance

10 *Formulation 2*

	<u>Ingredient</u>	<u>% w/w</u>
	Dicalcium phosphate dihydrate	50.0
	Sorbitol	10.0
	Glycerol	10.0
15	Sodium carboxymethyl cellulose	1.0
	Sodium lauryl sulphate	1.5
	Sodium lauroyl sarcosinate	0.5
	Flavour	1.0
	Sodium saccharin	0.1
20	Sodium monofluorophosphate	0.3
	Chlorhexidine gluconate	0.01
	Dextranase	0.01
	CPP-ACFP	2.0
	Water	balance

25 *Formulation 3.*

	<u>Ingredient</u>	<u>% w/w</u>
	Dicalcium phosphate dihydrate	50.0
	Sorbitol	10.0
	Glycerol	10.0
30	Sodium carboxymethyl cellulose	1.0
	Lauroyl diethanolamide	1.0
	Sucrose monolaurate	2.0
	Flavour	1.0
	Sodium saccharin	0.1

Sodium monofluorophosphate	0.3
Chlorhexidine gluconate	0.01
Dextranase	0.01
CPP-ACFP	5.0
5 Water	balance

Formulation 4

<u>Ingredient</u>	<u>% w/w</u>
Sorbitol	22.0
Irish moss	1.0
10 Sodium Hydroxide (50%)	1.0
Gantrez	19.0
Water (deionised)	2.69
Sodium Monofluorophosphate	0.76
Sodium saccharine	0.3
15 Pyrophosphate	2.0
Hydrated alumina	48.0
Flavour oil	0.95
CPP-ACFP	1.0
sodium lauryl sulphate	2.00

20 Formulation 5.

<u>Ingredient</u>	<u>% w/w</u>
Sodium polyacrylate	50.0
Sorbitol	10.0
Glycerol	20.0
25 Flavour	1.0
Sodium saccharin	0.1
Sodium monofluorophosphate	0.3
Chlorhexidine gluconate	0.01
Ethanol	3.0
30 CPP-ACFP	2.0
Linolic acid	0.05
Water	balance

Example 10

Mouthwash formulations.

Formulation 1 --

	<u>Ingredient</u>	<u>% w/w</u>
	Ethanol	20.0 -
	Flavour	1.0
5	Sodium saccharin	0.1
	Sodium monofluorophosphate	0.3
	Chlorhexidine gluconate	0.01
	Lauroyl diethanolamide	0.3
	CPP-ACFP	2.0
10	Water	balance

Formulation 2.

	<u>Ingredient</u>	<u>% w/w</u>
	Gantrez S-97	2.5
	Glycerine	10.0
15	Flavour oil	0.4
	Sodium monofluorophosphate	0.05
	Chlorhexidine gluconate	0.01
	Lauroyl diethanolamide	0.2
	CPP-ACFP	2.0
20	Water	Balance

Example 11

Lozenge formulation.

	<u>Ingredient</u>	<u>% w/w</u>
	Sugar	75-80
	Corn syrup	1-20
25	Flavour oil	1-2
	NaF	0.01-0.05
	CPP-ACFP	3.0
	Mg stearate	1-5
	Water	balance

30

Example 12

Gingival Massage Cream formulation.

	<u>Ingredient</u>	<u>% w/w</u>
	White petrolatum	8.0
	Propylene glycol	4.0

	Stearyl alcohol	8.0
	Polyethylene Glycol 4000	25.0
	Polyethylene Glycol 400	37.0 -
	Sucrose monostearate	0.5
5	Chlorohexidine gluconate	0.1
	CPP-ACFP	3.0
	Water -	balance

Example 13 Chewing Gum formulation.

	<u>Ingredient</u>	<u>% w/w</u>
10	Gum base	30.0
	Calcium carbonate	2.0
	Crystalline sorbitol	53.0
	Glycerine	0.5
	Flavour oil	0.1
15	CPP-ACFP	2.0
	Water	balance

Example 14 Dietary Supplement

CPP was added at 1.0% w/w of the diet of rachitic chickens to determine the ability of the CPP-ACP to provide bioavailable calcium for bone accretion. CPP-ACP at 1.0% w/w in the diet produced a 34% reduction in the incidence of growth plate abnormalities, a 17% increase in tibial ash and a 22% reduction in the cartilaginous growth plate in the animals which was significantly greater than the CPP alone (Table 4) indicating that the CPP-ACP is superior to the CPP in providing bioavailable dietary calcium and in facilitating bone accretion.

Table 4. Effect of 1.0% CPP-ACP addition to the diet of rachitic chickens on incidence of growth plate abnormalities, tibial ash and cartilaginous growth plate width

	% Growth Abnormalities %	% Tibial Ash %	Growth Plate Width (mm)
Control	53 ± 5	30 ± 2	5.4 ± 0.2
1.0% CPP	47 ± 9	30 ± 2	5.3 ± 0.2
1.0 % CPP-ACP	35 ± 3	35 ± 1	4.2 ± 0.2

It should be understood that while the invention has been described in detail for the purposes of clarity and understanding, the examples were for illustrative purposes only. Other modifications of the embodiments of
5 the present invention will be apparent to those skilled in the art of molecular biology, dental diagnostics, and related disciplines and are within the scope of the invention as described.

Dated this 13th Day of March 1997
10 The University of Melbourne
-and-
Victorian Dairy Industry Authority